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Dynamic developmental regulation of the large non-coding RNA associated with the mouse 7C imprinted chromosomal region

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Abstract

The mouse ortholog of the Prader–Willi/Angelman syndrome imprinted domain contains several paternal-specific transcripts and the maternally expressed gene encoding ubiquitin protein ligase E3A (*Ube3a*). A Large paternal Non-Coding RNA, encompassing *Snurf-Snrpn* exons and the *Ube3a* Antisense Transcript (*Ube3a-ATS*), has been recently characterized and named here *LNCAT*. Potential roles of *LNCAT* in imprinting, gene regulation, and disease are likely but have not been investigated. In order to establish the function(s) of *LNCAT*, we first determined its in vivo spatio-temporal expression pattern at the cellular level during development and in different adult brain tissues.

We show here that *LNCAT* is developmentally regulated, with transcript variants being specifically expressed through neuronal differentiation in postmitotic neurons. We demonstrate that the *LNCAT* and *Snurf-Snrpn* transcripts are independent although they share common exons. We show an absence of expression of *LNCAT* through gametogenesis and in early embryo excluding a role of *LNCAT* in the imprint establishment. We also report a range of observations that challenges the widely accepted model of imprinted gene silencing of *Ube3a*. Although these last data do not completely exclude that the *LNCAT* variants including "*Ube3a-ATS*"exons could repress the paternal allele of *Ube3a*, they do allow us to propose an alternative and consistent model.

Keywords: Non-coding RNA; Imprinting; Prader–Willi; Angelman; *Ube3a*; *Snrpn*

Introduction

Genomic imprinting in mammals results in the differential expression of maternal and paternal alleles of a small number of autosomal genes. The regulation of imprinted genes is initially determined by epigenetic modifications applied to the genomes during male or female gametogenesis. However, further modifications, established in early development and later during the differentiation steps of a specific type of cells, are necessary to read the imprinting mark allowing a monoallelic expression of these genes (Latham, 1999).

Among the molecular features that have highlighted the imprinting mechanism, the non-coding and antisense RNAs have been frequently found within imprinted gene clusters (Lee, 2003). Functional non-coding RNAs are implicated in regulating several epigenetic phenomena in *Arabidopsis*, *Drosophila*, Yeast or in X inactivation in mammals (Andersen and Panning, 2003; Mattick, 2004). Current thinking favors a general role of such non-coding (and antisense) RNAS in the in the *cis* regulation of imprinted genes (Delaval and Feil, 2004).

One of the most studied imprinted regions is the one involved in the Prader–Willi (Goldstone, 2004) and Angelman syndromes (Clayton-Smith and Laan, 2003); both are distinct neurogenetic disorders resulting from loss of expression of genes located in the 15q11–q13 region. PWS is caused by the loss of paternally expressed genes not

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yet clearly identified and located in a 4 megabase DNA interval. AS is caused by the loss or inactivation of the maternal *UBE3A* allele (Kishino et al., 1997; Matsuura et al., 1997). *UBE3A* encodes an E6-AP ubiquitin protein ligase, involved in the ubiquitin–proteasome protein degradation pathways (Nawaz et al., 1999). Tissue-specific expression of *UBE3A/Ube3a* in human (Rougeulle et al., 1997; Vu and Hoffman, 1997) and mouse (Albrecht et al., 1997) shows paternal silencing in brain but not in somatic tissues. There is evidence that *Ube3a* is imprinted in neurons only (Yamasaki et al., 2003). Region-specific data in mouse reveal that *Ube3a* imprinting results in complete silencing of the paternal allele in very specific regions only, the most evident being the cerebellar Purkinje cells and the CA3 hippocampal neurons (Albrecht et al., 1997; Jiang et al., 1998; Miura et al., 2002). A much stronger bias towards the expression of the maternal allele with a low level of expression of the paternal allele is, however, detectable more globally in brain (Miura et al., 2002; Chamberlain et al. 2001) (Landers et al, submitted for publication). The latter findings suggest that the paternal allele is also imprinted but not silenced in those regions.

The 7c mouse chromosomal region is the orthologous region of the human PWS/AS locus. The genes, their organization, and their imprinting regulation are similar in both species. Imprinting mechanism share also conserved elements in both regions. Remarkably, a *cis*-acting DNA sequence called imprinting center (IC), including *SNRPN*/*Snrpn* exon 1, has been functionally characterized. Its paternal deletion in mouse silences the paternal expressed genes as it has been observed in humans (Buiting et al., 1995; Sutcliffe et al., 1994; Yang et al., 1998). This element is required for the gametic establishment and the postzygotic maintenance of paternal imprint (Bielinska et al., 2000; Geuns et al., 2003; Lucifero et al., 2004).

In addition to the PWS/AS-IC that controls imprinting at a regional level, other imprinting regulatory elements are shared between mouse and human (Watrin et al., 2005). Recently, we have described (Landers et al., 2004) the organization of a large transcriptional unit encompassing the PWS imprinting center, the gene encoding small nuclear ribonucleoprotein N and its upstream reading frame (*Snurf-Snrpn*), *Ipw* and the *Ube3a* antisense transcript (*Ube3a-ATS*). This large transcriptional unit spans more than one megabase and encodes for complex alternatively spliced transcripts that appear to function as non-coding RNAs. All these transcripts are exclusively expressed from the paternal allele (Landers et al., 2004) (Fig. 1). We have named this transcriptional unit *LNCAT* since no specific name was assigned to it before. Furthermore, it is important to make clear that *Ube3a-ATS* designates only one part, probably associated to specific variants, of this transcript. Our previous findings suggest that these brain-specific non-coding transcripts initiate in exons (exons U) that are distributed in a 500-kb region upstream of *Snurf-Snrpn*. These data are consistent with an evolutionary conserved genomic organization for this transcriptional unit between human (Runte et al., 2001) and mouse and support the hypothesis of a conserved function of these non-coding antisense transcripts (Landers et al., 2004). This function has not yet been investigated. Nonetheless, distinct roles have been proposed: (1) to serve as a host for the snoRNAs (Landers et al., 2004; Runte et al., 2001), (2) to silence the paternal expression of *Ube3a* via the *Ube3a* antisense transcript (*Ube3a-ATS*) (Chamberlain and Brannan, 2001; Rougeulle et al., 1998), and (3) to establish and/or to maintain the parental imprint in the "PWS region" (Runte et al., 2001). Lastly, *LNCAT* could result from a leakage of the RNA transcription machinery and its biological function is questionable.

In this study, we address the question of the validity of these hypothetical roles by investigating the developmental and brain-structure expression patterns of *LNCAT* compared to various probes.

The investigation of the spatio-temporal regulation/expression of *LNCAT* in vivo, at the cellular level, is crucial in terms of determining its function. *LNCAT* encodes overlapping non-coding transcripts corresponding to different variants (Landers et al., 2004) that might have distinct functions. In order to clarify the regulation and specific functions of this large non-coding transcriptional unit (*LNCAT*), we investigate here the detailed expression pattern of several *LNCAT* exons (U exons, *Ipw BE*, *Ipw BC*, *Ube3a-ATS*) compared with the *Snurf-Snrpn* transcript and the snoRNAs, during gametogenesis, early development, and adulthood.

Materials and methods

Mouse strains

Adult organs come from C57BL/6 mice (CERJ, France) and embryos come from CD1 strain (Charles River Laboratories).

RT-PCR and Northern blot analysis

Total DNase-treated RNA was isolated from embryos and adult tissues by using TRIzol (Life Technologies) or RNAzol (Tel-test, Inc., Friendswood, TX). Reverse transcription reactions (+RT) were performed with 2.5 μg of RNA and Superscript™ II RNaseH$^-$ reverse transcriptase (Life Technologies) using random hexamer primers. A 2.5-μg aliquot of total RNA was incubated in a similar manner but without reverse transcriptase (−RT) as a control. One twentieth of the +RT or −RT was used in PCR reactions using *Taq* Polymerase (Promega or Invitrogen). Oligonucleotides were U consensus (5′-TCAGTGCAGCAGGTCCTGCT-3′); MBII52-F (5′-CTGGAAGGCAT-TTCGTCC-3′); MBII52-R (5′-CCCAAGGAGTCAACGGAC-3′); MBII85-F (5′-CCAGGCCCTTCGGGACA-3′); MBII85-R (5′-TGTGCCTGACGCCCATA-3′); *Ube3a-ATS* L-1R (5′-ATCG-

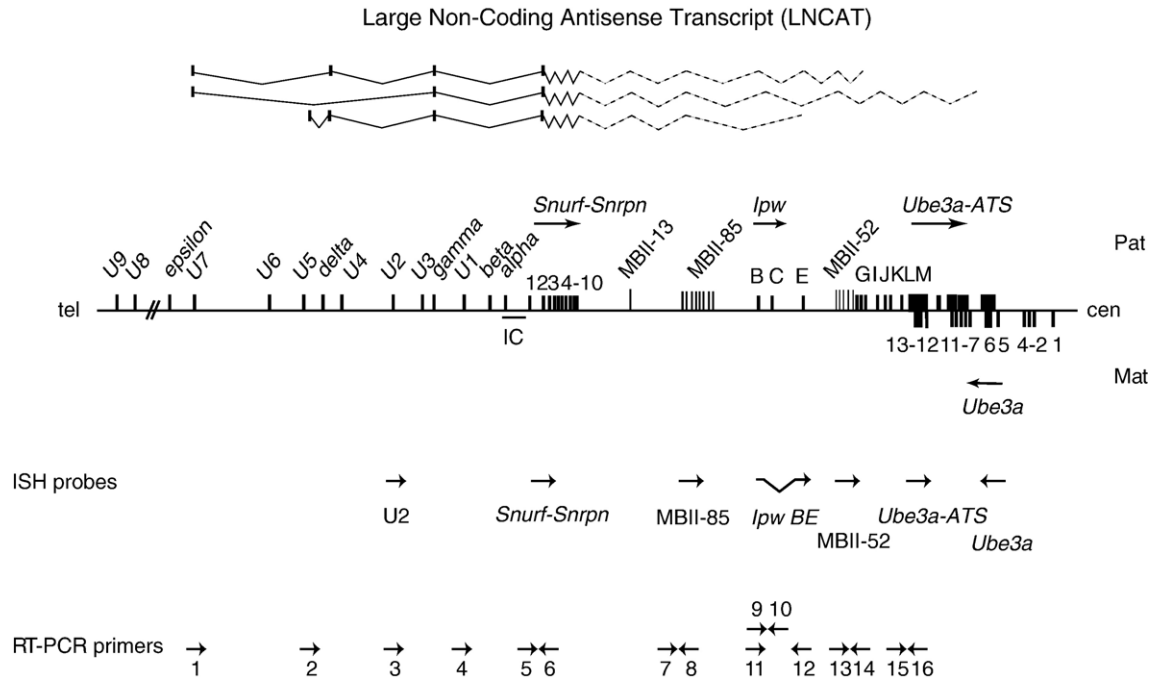


Fig. 1. Schematic map of the mouse chromosome 7C region encompassing the large non-coding antisense transcriptional unit, extending from the U exons to *Ube3a*. The variants of the Long Non-Coding Antisense Transcript (*LNCAT*) are initiated at different U exons and spliced to alternative *LNCAT* exons including *Snurf-Snrpn*, *Ipw*, and *Ube3a-ATS* exons. Some potential splice variants are represented above the map. Designation of *Ipw* and *Ube3a-ATS* exons is as in Landers et al. (2004). The snoRNAs MBII-13, MBII-85, and MBII-52 that are encoded by intronic sequences are processed from *LNCAT*. Paternally or maternally expressed exonic and intronic sequences are represented respectively by black boxes/lines above or below the chromosome schematic line. The positions of RT-PCR primers used in the studies are indicated below the map. Thus, in the 5′ part, we used primers to amplify exon U/Snrpn exon 3 (specific primers for different U exons have been designed), while in the central region, we analyzed Ipw exon B–exon C or Ipw exon B–exon E (Landers et al., 2004). In the part overlapping *Ube3a*, we used two pairs of primers to amplify *Ube3a-ATS* exons: exon L–exon M and primers in exon M, overlapping the *Ube3a* intron 12 (not indicated). We also performed the analysis on the MBII-52 and MBII-85 snoRNAs. The ISH riboprobes selected for expression studies are: exon U2, which should detect the expression of all U exons since these latter share more than 90% of sequence identity, Ipw exons B–E and the MBII-52 and MBII-85 snoRNAs. For *Ube3a-ATS*, we chose a probe in exon M of the antisense transcript, referred as *Ube3a-ATS* in the text. The *Snurf-Snrpn* transcript was analyzed with a *Snurf-Snrpn* exons 1–4 probe. cen, centromere; IC, Imprinting Center; tel, telomere; Pat, Paternal chromosome; Mat, Maternal chromosome. Primers referred as numbers are 1, U7; 2, U5; 3, U2; 4, U1; 5, *Snurf* exon 1; 6, *Snurf* exon 3; 7, MBII-85-F; 8, MBII-85-R; 9, Ipw B–F; 10, Ipw C–R; 11, Ipw B–1R; 12, Ipw E–2F; 13, MBII-52-F, 14, MBII-52-R; 15, *Ube3a-ATS* L-1R; 16, *Ube3a-ATS* M-6F bis.

AAAAAACAAGCTATCCAATC-3′); *Ube3a-ATS* M-F-bis (5′-CCAGGCTGTAAT ACATCTGTCGA-3′); Gabrα5-F (5′-ATTCACCAGGATCTTGGACGG-3′); Gabrα5-R (5′-AGCCTCAGCAGCTTGTTGGG-3′); *Pcp2*-F (5′-GTGTAACAGTTAATTCCCTGCC-3′); *Pcp2*-R (5′-TGGCTAGA-ACTCTCAAGGAGC-3′). Specific annealing temperatures and extension times are available upon request. The oligonucleotide primers and PCR conditions for Exon U1–*Snurf* exon 3 and Exon U2–*Snurf* exon 3 (Bressler et al., 2001), *Gapdh* (Yang et al., 1998), *Hprt* (Boccaccio et al., 1999), *Ipw BC*, *Ipw BE*, exon U5–*Snurf* exon 3, exon U7–*Snurf* exon 3 (Landers et al., 2004), and *Snurf* exons 1–3 (Tsai et al., 1999) have been described previously.

Northern blots were performed on 15 μg of total DNase-treated RNA as previously described (Jay et al., 1997) and were probed with *Snurf-Snrpn* exons 1–4, *Ube3a-ATS*, *Ipw BE*, and U2 PCR products used for in situ hybridization. As a control, mouse, *β-Actin* probe was amplified from cDNA with primers: *β-Actin*-F: 5′-GTG GGC CGC TCT AGG CAC CAA-3′ and *β-Actin*-R: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′.

In situ hybridization and immunohistochemistry

Embryos were removed from the uterus of timed pregnant CD1 mice and dissected in RNAse free phosphate-buffered saline (PBS), fixed overnight in 4% paraformaldehyde (PFA) in PBS, post-fixed overnight in 15% sucrose in PBS, embedded in OCT compound, and frozen with liquid nitrogen vapors. C57BL/6 adult brain were dissected and directly frozen on a copper plate on liquid nitrogen. Frozen embryos and adult brains were sectioned with a thickness of 12 μm in a cryostat. Embryo sections were air dried and frozen until use whereas adult brain sections were briefly air dried, fixed in 4% PFA for 30 min, dehydrated and frozen until use. Sections were washed in 1× PBS, treated with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8), post-fixed in 4% PFA, acetylated and further washed in PBST (1× PBS, 0.1% Tween-20) before pre-hybridization for 1 h and overnight hybridization steps. Pre-hybridization, hybridization, and post-hybridization washes were performed at 70°C. Hybridization was

performed in 50% formamide, 5× SSC, 5× Denhardt's solution (Sigma), 0.5 mg/ml herring sperm DNA, 0.25 mg/ml yeast RNA. Washes were performed in 0.2× SSC. Antisense riboprobes were transcribed with T7 RNA Polymerase, and the digoxygenin label was detected using anti-digoxygenin Fabs coupled to alkaline phosphatase (Roche) and NBT/BCIP. No signal was detected with the sense control probes. *Necdin* probe has been previously described (Andrieu et al., 2003). MBII-52 and MBII-85 probes are synthetized from pUC18/T7 vector, linearized by *Bam*HI (Gift from J. Cavaillé). Other probes were synthetized from PCR products using T7-promoter containing primers: *Snurf-Snrpn* probe recognizes the first four exons of *Snurf-Snrpn* that are unrelated to *Snrpb*; primers were 5′-GAGGAGTGATTTGCAACGC-3′ and 5′-TGCTGTTCCACAATAGCCG-3′. *Ube3a-ATS* probe was designed against intron 12 of *Ube3a*; primers were 5′-CTTGATAACGTCTGTACTTCTG-3′ and 5′-ACTTTGTACCCACTGTAACC-3′. *Ipw BE* probe was synthetized from Ipw B-1R and Ipw E-2F primers. U2 probe should detect the expression of all U exons since these latter share more than 90% of sequence identity; it was synthetized from 5′-AGTCAATTCAGTGCAGCAGG-3′ and 5′-CTTGGTTGCTGCATTGCC-3′ primers. *Ube3a* probe recognizes the exon 6; primers were 5′-TATCTGGAAATGGCGTTGC-3′ and 5′-GCTCCTGAAGTGTTAATTCGC-3′. The *Oct-4* control probe was synthetized from *Oct-4*-991: 5′-CATGCATTCAAACTGAGGCA-3′ and *Oct-4*-1200: 5′-AGAACAAAATGATGAGTGAC-3′ primers. Specific annealing temperatures and extension times are available upon request.

The following primary antibodies were used: rabbit polyclonal anti-Neurofilament M (Chemicon) and mouse monoclonal anti-calbindin (SIGMA). Secondary antibodies were Alexa 488-conjugated goat anti-rabbit IgG antibody and peroxidase conjugated goat anti-mouse IgG antibody.

Results

The transcription of the *LNCAT* unit is complex and generates numerous transcripts by alternative splicing and from alternative 3′ end usage (Landers et al., 2004). The complexity of these transcriptional variants in terms of structure and sequence (many of the exons are repeat sequences) makes it difficult to perform RT-PCR amplification in the region extending from U exons to the *Ube3a-ATS* exons that overlap *Ube3a*. Only connection by RT-PCR has been possible between proximal exons (Landers et al., 2004). In our previous work using an in vitro cell culture system, we have strongly suggested that *LNCAT* initiates at the U exons (Landers et al., 2004).

To investigate the spatio-temporal expression pattern of *LNCAT*, we selected the most representative RT-PCR products in order to cover exons from the 5′, central, and 3′ regions of *LNCAT*. In parallel, we have analyzed the *Snurf* exon 1–exon 3, *Ube3a*, and a serial of control transcripts. A series of RNA sense and antisense probes were tested by in situ hybridization (ISH) on mouse tissue sections. Only the probes with target-specific signals (the sense control probes giving no signal) have been selected for further analysis using ISH (Fig. 1).

The expression pattern of LNCAT, snoRNAs, and Snurf-Snrpn in adult tissues

In order to confirm that *LNCAT* is distinct from the *Snurf-Snrpn* transcript, we used different approaches. By RT-PCR analysis, we show that several exons of the *LNCAT* are expressed in brain at birth (P0) as well as in adulthood but not in the other tissues examined (liver, placenta, intestine, kidney, heart) (Fig. 2A). The expression of *LNCAT* appears to be correlated to that of the U exon-containing transcripts while the *Snurf* transcript is expressed in all tissues tested (Fig. 2A). By Northern blot analysis, *Snurf-Snrpn* displays strong expression in adult and P4 brain and a lower expression in other adult tissues (Fig. 2B-a). The signal detected is a discrete band of 1.6 kb, corresponding to the size of *Snurf-Snrpn* bicistronic transcript (Gray et al., 1999). These results indicate that although the *Snurf-Snrpn* transcription is ubiquitous, a higher level of expression is observed in brain. In parallel, Northern blots analyzed with a mixture of exon U, exon M, and *Ipw BE* probes revealed a hybridization smear in adult and P4 brain only, suggesting that these antisense exons belong to a large variety of alternative transcripts but are independent of *Snurf-Snrpn* transcripts (Fig. 2B-b). Interestingly, the exon U probe does not hybridize to the discrete 1.6 kb fragment corresponding to the *Snurf-Snrpn* bicistronic transcript suggesting that the U exons should be rarely used as alternative exons to produce a *Snurf-Snrpn* bicistronic transcript.

These data strongly suggest that *LNCAT* and *Snurf-Snrpn* are independent transcripts.

LNCAT and snoRNAs are expressed in neurons from various adult brain regions

RNA in situ hybridizations on mouse adult brain sections were performed in order to define the brain territories expressing the different exons from *LNCAT*, the snoRNAs and *Snurf-Snrpn*. Although all are expressed in various brain regions including the cortex, hypothalamus, hippocampus, cerebellum, and olfactory bulb, the expression levels are variable. Regional differences in expression level are, nonetheless, detected between *Snurf-Snrpn*, on the one hand, and the snoRNAs and *LNCAT* exons on the other (Fig. 3A). Thus, as shown in hippocampus, *Snurf-Snrpn* is highly expressed in the CA2 and CA3, with a lower level in the dentate gyrus and CA1 (Fig. 3B-b). In contrast, exon U2, MBII-85, *Ipw BE*, and

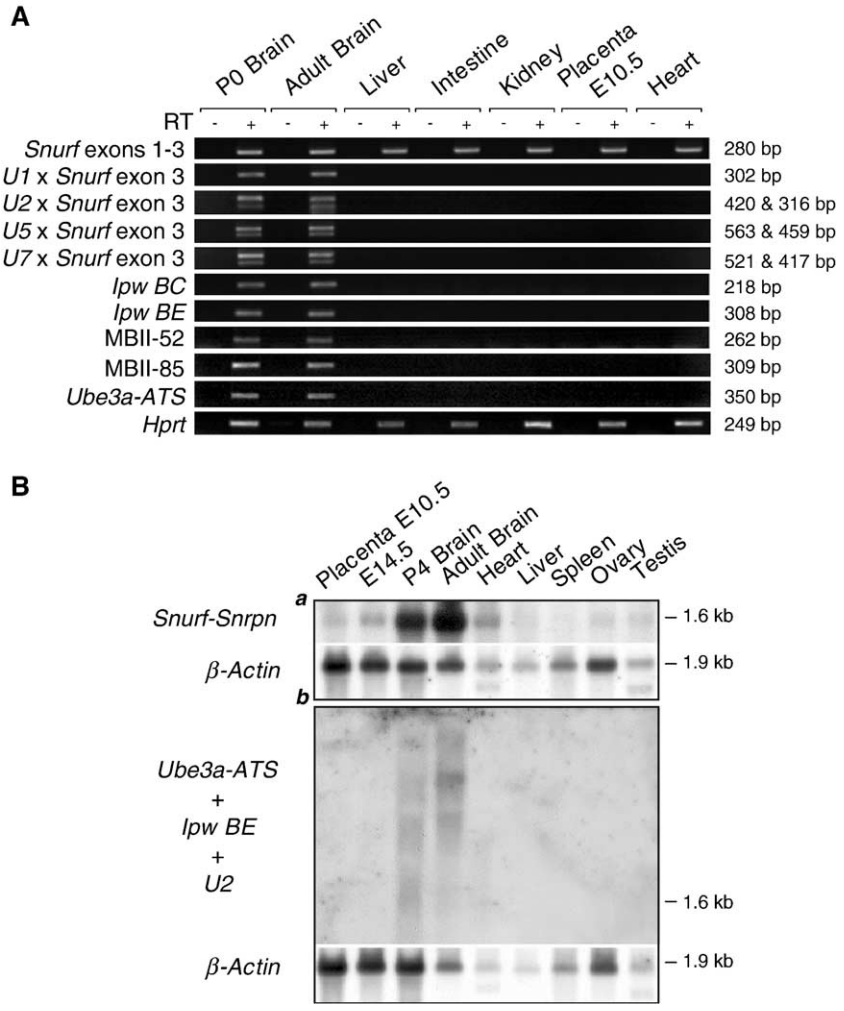


Fig. 2. RT-PCR and Northern blot analysis of *LNCAT*, snoRNAs, and *Snurf-Snrpn* transcripts in mouse adult tissues. (A) RT-PCR analyses in adult mouse tissues. Several specific primers pairs were used to detect different exonic regions of the *LNCAT*: *U* exons (U1/U2/U5/U7)–*Snurf* exon 3, *Ipw* exons B–C (*Ipw BC*), *Ipw* exons B–E (*Ipw BE*), and *Ube3a-ATS* exons L–M (*Ube3a-ATS*). RT-PCR products for *LNCAT* as well as for the snoRNAs MBII-85 and MBII-52 were detected only in brain (P0 and adult brain) whereas RT-PCRs *Snurf-Snrpn* products were detected in all the tissues examined (liver, intestine, placenta, and heart). RNA sample integrity was controlled by amplification of an *Hprt* RT-PCR product. (+), with reverse transcriptase (RT); (−), without RT. (B) Northern blot analysis of mouse embryonic and adult tissues. Hybridizations were performed with a *Snurf-Snrpn* probe (a) or a mixture of *U2*, *Ipw BE*, and *Ube3a-ATS* probes to detect LNCAT (b). No smear is detected with *Snurf-Snrpn* probe. Northern blots were re-hybridized with a mouse *β-Actin* probe in order to quantify RNA loading. We noticed a downloading for heart, liver, and testis RNAs; however, an exposition over a longer period does not allow to detect a signal for LNCAT in these tissues.

Ube3a-ATS present a complementary expression pattern with relatively higher expression in dentate gyrus and CA1 as compared to the CA2 and CA3 structures (Figs. 3B-a,c,d,e). No hybridization signal was detected with all the sense probes corresponding to the antisense probes used in these experiments (data not shown).

The complexity of the *LNCAT* due to the multiple variants initiated at different U exons (Landers et al., 2004; Fig. 1) raises the question whether there is a region-specific regulation of some variants potentially linked to specific U exons. To address this difficulty, we performed RT-PCR using specific primers designed for each exon U. We used RNAs extracted from olfactory bulb, cortex, hippocampus, and cerebellum from mouse adult brain. The quality and tissue (region) specificity of cDNAs was controlled using primers for genes specifically expressed in some of these regions such as the gamma-aminobutyric acid receptor, subunit alpha 5, (Gabrα5), and the Purkinje cell protein 2 (*Pcp2*). We observe that all U exon-containing transcripts are expressed in the different dissected brain regions thus demonstrating no correlation between a brain territory and specific exon U-containing transcript expression (Fig. 3C).

In order to confirm an in vivo neuronal expression of the *LNCAT*, we performed several double labeling experiments using an anti-Neurofilament (NF) antibody as a neuronal marker and specific RNA probes for *LNCAT*. The neuronal marker was detected by immunohistochemistry whereas the different exons from the

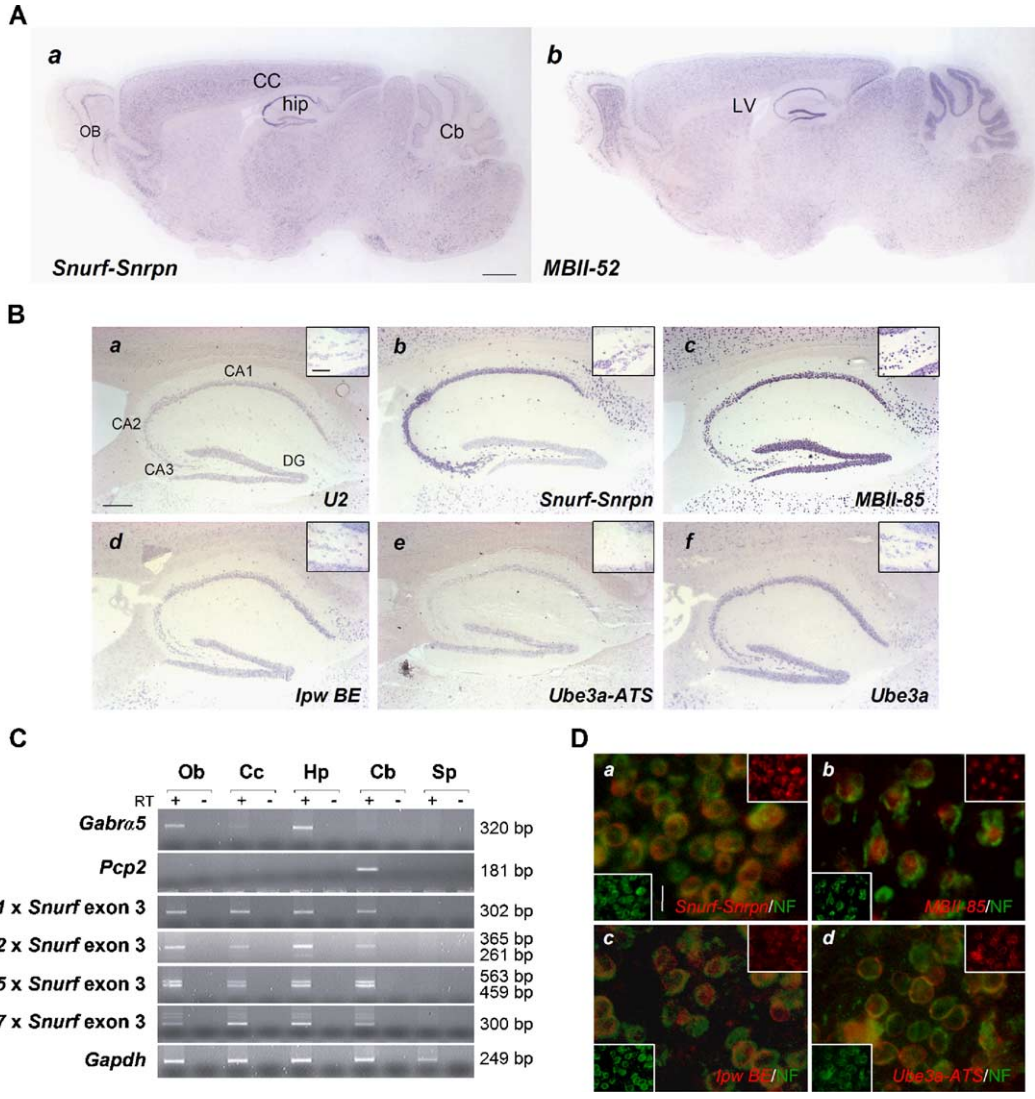


Fig. 3. Expression analysis of *LNCAT*, snoRNAs, *Snurf-Snrpn*, and *Ube3a* transcripts in adult mouse brain. (A–B) In situ hybridization was performed on sagittal sections of adult mouse brain. (A) Low magnifications of the entire brain with *Snurf-Snrpn* (a) and MBII-52 (b) probes. (B) Higher magnifications of the hippocampus (a–f). *LNCAT* is detected with either riboprobes *U2* (a), *Ipw BE* (d) or *Ube3a-ATS* (e) and is observed predominantly in the CA1 and dentate gyrus regions, as are the snoRNAs MBII-85 (c) and *Ube3a* transcripts (f). In contrast, *Snurf-Snrpn* transcripts (b) are detected mostly in the CA2 and CA3 regions. Higher magnifications in the top right hand corners show the CA3 region of the hippocampus. Noticeably, *Ube3a-ATS* is not expressed at all in this structure. CA, Cornus Ammonis; Cb, cerebellum; CC, cerebral cortex; DG, dentate gyrus; hip, hippocampus; LV, lateral ventricule; OB, olfactory bulb. (C) RT-PCR analysis of U exon-containing transcripts in mouse brain subregions. Primers pairs used for U exons–*Snurf* exon 3 were as described in Fig. 2A. Gabrα5 and *Pcp2* were used as specific control probes for subregions: Gabrα5 is expressed only in cerebral cortex, hippocampal formation, and olfactory bulb granular layer whereas *Pcp2* is restricted to Purkinje cells of cerebellum. RNA sample integrity was controlled by amplification of a *Gapdh* RT-PCR product. Asterisks indicate different RT reactions. (+), with reverse transcriptase (RT); (−), without RT. Ob, olfactory bulb; Cc, Cortex; Hp, Hippocampus; Cb, Cerebellum; Sp, Spleen. (D) Neuronal-specific expression of *LNCAT*, snoRNAs, and *Snurf-Snrpn* in the mouse cerebral cortex. Simultaneous detection of *LNCAT* with *Ipw BE* (c) or *Ube3a-ATS* (d) riboprobes, MBII-85 snoRNAs (b) and *Snurf-Snrpn* transcripts (a) by in situ hybridization (in the top right hand corner, in red) and of the Neurofilament (NF) protein, a neuronal differentiation marker by immunohistochemistry (in the bottom left hand corner, in green) was performed on coronal sections of adult brain. The merged images show a strict colocalization of each transcript with the Neurofilament protein. Scale bar: 1 mm (A), 250 μm (B), 80 μm for higher magnifications of CA3 (B), 10 μm (D).

LNCAT were detected by ISH. This combined approach allows the colocalization of all the distinct *LNCAT* probes with NF in the different brain regions (Fig. 3D).

As expected, the snoRNAs are clearly localized in the nucleus. The other ISH probes (exon U, *Snurf*, *Ipw BE*, *Ube3a-ATS*) reveal mainly cytoplasmic hybridization with some nuclear localization observed for the *Ipw BE* and *Ube3a-ATS* probes.

The expression pattern of Ube3a-ATS relative to that of Ube3a

The results of Fig. 3 demonstrate the widespread expression pattern of LNCAT, including Ube3a-ATS exons, in the brain.

In order to decipher the role of the *LNCAT* in the imprinting regulation of *Ube3a*, we focused our analysis on

brain regions where the allele-specific expression of *Ube3a* has previously been investigated. We decided to focus our study on the Purkinje cells and the neurons of the CA3 region of hippocampus where paternal *Ube3a* is silenced with exclusive maternal expression. Identification of Purkinje cells was done on morphological criteria and using an anti-Calbindin antibody as a specific marker (Celio, 1990). We performed a double labeling experiment using anti-calbindin antibody by immunohistochemistry and different *LNCAT* RNA probes by ISH on cerebellar sections of adult mouse brain. We observed a clear expression of *Ube3a* and U exons, *Snurf-Snrpn*, MBII-52 and -85, and *Ipw BE* in the Purkinje cells but *Ube3a-ATS* is not expressed in these cells (Fig. 4A). The lack of expression of *Ube3a-ATS* in the Purkinje cells most likely results from the absence of specific *LNCAT* variants since other *LNCAT* exons are expressed. In order to support a correlation between the lack of this specific antisense variant and the absence of paternal expression of *Ube3a*, we analyzed the expression patterns of *Ube3a-ATS* in the CA3 region of hippocampus. Again, we did not detect any signal with the *Ube3a-ATS* probe in these cells (Fig. 3B). We also designed another RNA probe corresponding to exons L–M, overlapping the *Ube3a* 3′ end, and obtained the same results (data not shown).

These findings suggest that, among the transcript variants encoded by the *LNCAT*, only the subset that overlaps the *Ube3a* gene could be involved in the imprinting regulation of *Ube3a* since other *LNCAT* transcripts are present in neurons whatever the imprinted status of the paternal allele of *Ube3a*. Other key results of our analysis are that: (1) in the few regions where the paternal allele of *Ube3a* is silenced, *Ube3a-ATS* transcript(s) overlapping the *Ube3a* gene is (are) absent and; (2) in the other brain regions, where there is a strong bias of expression of maternal versus paternal allele, Ube3a-ATS is expressed. We also demonstrate an expression of *Ube3a-ATS* in the optic chiasma and anterior commissure regions (Fig. 4B) where *Ube3a* displays biallelic expression (Albrecht et al., 1997; Jiang et al., 1998). It should be noted that these structures (chiasma optic and anterior commissure) contain only axons and oligodendrocytes.

The LNCAT transcriptional unit is developmentally regulated

The expression of the *LNCAT* through development was first studied by RT-PCR using RNAs extracted from whole embryos at different developmental stages. All exons U tested, *Ipw BC*, *Ipw BE*, MBII-52, MBII-85, and *Ube3a-ATS* are expressed from 10.5 days post-coitum (dpc) embryonic stage and have not been detected earlier (Fig. 5A). In contrast, *Snurf-Snrpn* is detected in blastocysts in 7.5 dpc embryos and in all later development stages tested (Figs. 5A and B). A semi-quantitative RT-PCR analysis shows a peak of expression at 14.5 dpc for all exons tested, including *Snurf-Snrpn* (data not shown).

In 7.5 dpc embryo, no *LNCAT* expression was detected by ISH with U exons or the snoRNAs probes whereas *Snurf-Snrpn* was expressed in the embryonic ectoderm (Fig. 5B). In 9.5 dpc embryos, we observe *Snurf-Snrpn* expression in mitotic cells of the neural tube. At 10.5 dpc, it continues to be expressed in few mitotic neurons but mainly appears in the first appearing postmitotic neurons (data not shown). *Snurf-Snrpn* expression is restricted to the nervous system from 10.5 dpc until 14.5 dpc embryonic stages (later embryonic stages have not been tested) although, in adulthood, it is expressed in other tissues (Figs. 2 and 5C). All *LNCAT* exons tested from 10.5 dpc until 14.5 dpc embryos are exclusively expressed in the nervous system with a pattern similar to that previously reported for *Necdin*, suggesting an expression restricted to postmitotic neurons

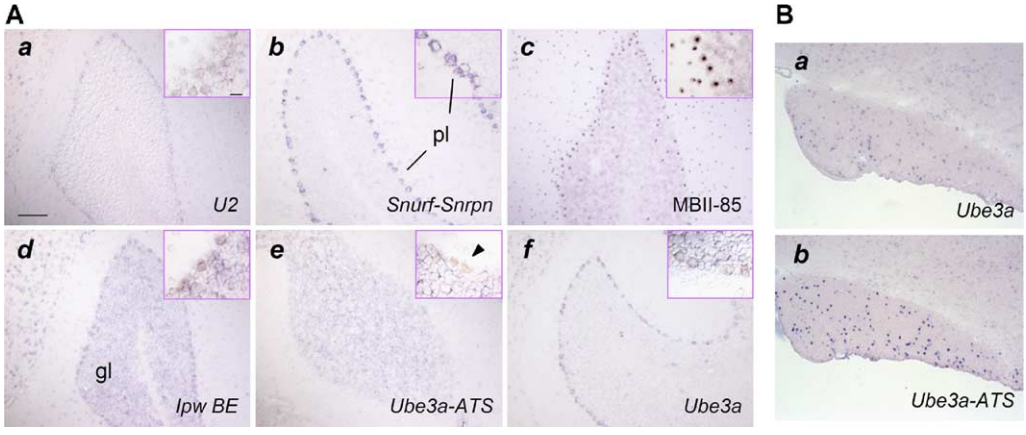


Fig. 4. Expression of *LNCAT*, snoRNAs, *Snurf-Snrpn*, and *Ube3a* in the mouse cerebellum and optic chiasma. (A) In the cerebellum, in situ hybridization was performed with *U2* (a), *Snurf-Snrpn* (b), MBII-85 (c), *Ipw BE* (d), *Ube3a-ATS* (e), and *Ube3a* (*f*) riboprobes. Double labeling experiments were performed with an anti-calbindin antibody, a specific marker of Purkinje cells and with U2, MBII-85, *Ipw BE*, *Ube3a-ATS*, and *Ube3a* riboprobes. Anti-calbindin antibody labeling can be visualized as the brown-colored accumulation in the right hand corner of the higher magnifications. Signals are detected in Purkinje cells with all the riboprobes except that for *Ube3a-ATS* (arrowhead). Note that although *Ube3a-ATS* is not detected in the Purkinje cells, it is detected in the granule cell layer. (B) In the optic chiasma, in situ hybridizations show that *Ube3a* (a) and *Ube3a-ATS* (b) are both expressed in axons of optic chiasma neurons. Gl, granule cell layer; Pl, Purkinje cell layer. Scale bar (shown in A-a) 100 μm (A, B); higher magnifications (shown in A-a), 20 μm.

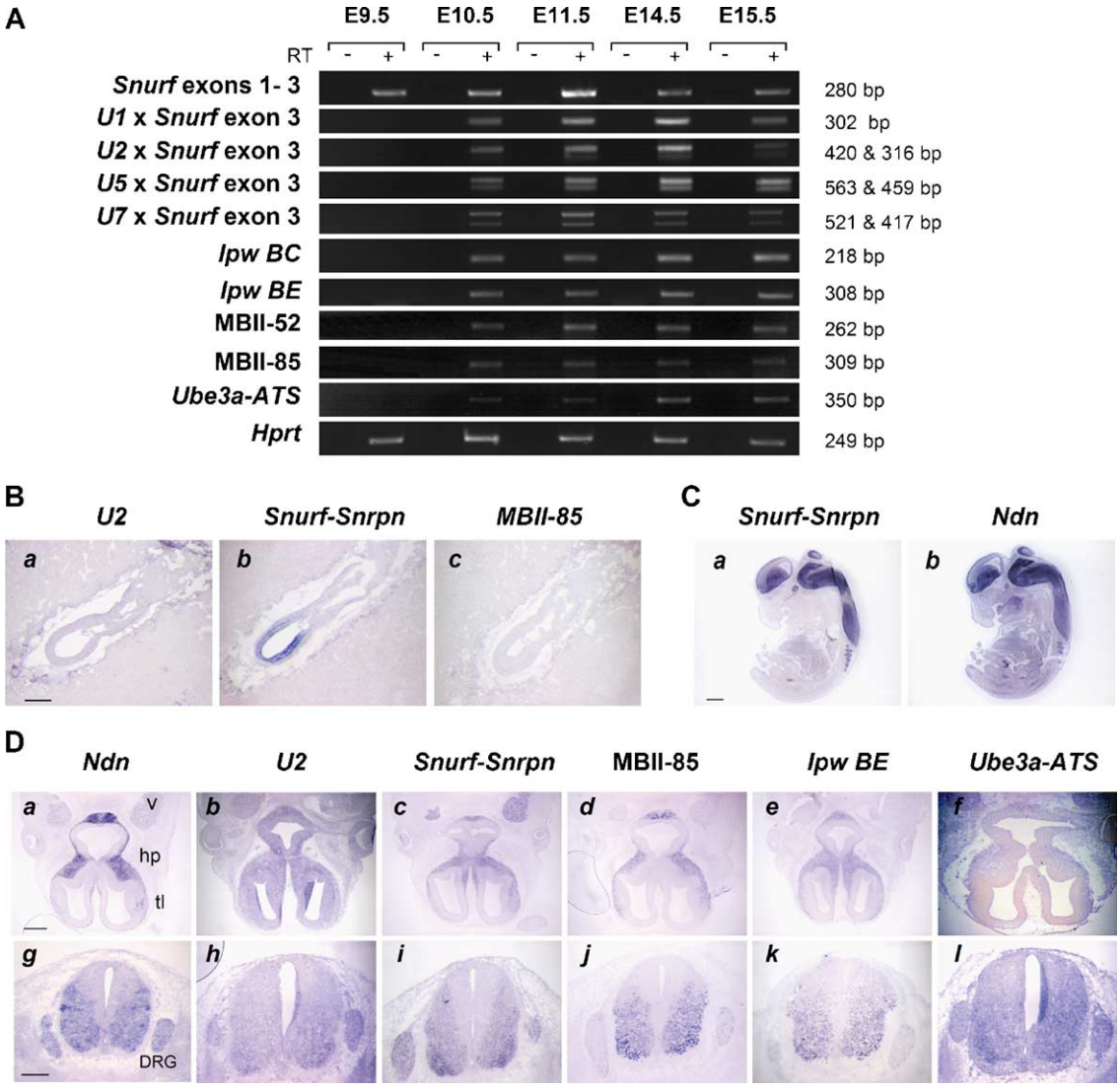


Fig. 5. Expression of *LNCAT*, snoRNAs, and *Snurf-Snrpn* transcripts through mouse embryogenesis. (A) RT-PCR expression analyses of *LNCAT*, snoRNAs, and *Snurf-Snrpn* in E9.5, E10.5, E11.5, E14.5, E15.5 mouse embryos. Primer pairs used were as described in Fig. 2A. RNA integrity was controlled by amplification of an *Hprt* RT-PCR product. (+), with RT; (−), without RT. (B–D) In situ hybridization analyses of *LNCAT*, snoRNAs, and *Snurf-Snrpn* transcripts through mouse embryogenesis. (B) Sagittal sections of 7.5 dpc embryos reveal that *Snurf-Snrpn* (b) is highly expressed in the embryonic ectoderm, whereas neither *U2* (a) nor MBII-85 (c) are expressed. (C) Sagittal sections of 12.5 dpc embryos reveal that *Snurf-Snrpn* (a) is expressed exclusively in the nervous system, as is the *Ndn* gene (b). (D) Transverse sections of 12.5 dpc embryos show that signals corresponding to U2 (b, h), *Ipw BE* (e, k), *MBII-85* (d, j), *Ube3a-ATS* (f, l), and *Snurf-Snrpn* (c, i) transcripts are detected in regions where the *Ndn* gene (a, g) is expressed: in the hypothalamus and telencephalon (a, b, c, d, e, f) and in the mantle layer of the spinal chord (g, h, i, j, k, l). tl, telencephalon; hp, hypothalamus; v, trigeminal (V) ganglion; DRG, dorsal root ganglion. Scale bar, 200 μm (B), 1 mm (C), 500 μm (D, a–f), 200 μm (D, g–l).

(Fig. 5C; Andrieu et al., 2003). We more precisely compared the expression pattern of *Necdin* to that of exon U, MBII-52, MBII-85, *Ipw BE*, *Ube3a-ATS*, and *Snurf-Snrpn* transcripts at 12.5 dpc embryonic stage and in the same region of the nervous system. Predominant expression of these transcripts is observed in ventral parts of the neural tube, specifically in the marginal areas where differentiating neurons reside, a pattern similar to that reported for *Necdin* (Figs. 5C and D) (Andrieu et al., 2003). Nevertheless, some differences of expression are observed in the peripheral nervous system where *Snurf-Snrpn*, but not MBII-85 or MBII-52, is expressed in cranial and dorsal root ganglia (Fig. 5D). No hybridization signal was detected with all the sense probes corresponding to the antisense probes used in these experiments (Supplementary Data).

LNCAT and Snurf-Snrpn are not transcribed through gametogenesis

We next wished to study whether the *LNCAT* and/or the *Snurf-Snrpn* transcript might play a structural role for establishing the imprinting pattern of the entire region. These transcripts could function in the opening of chromatin structure of the IC (PWS-IC) throughout male

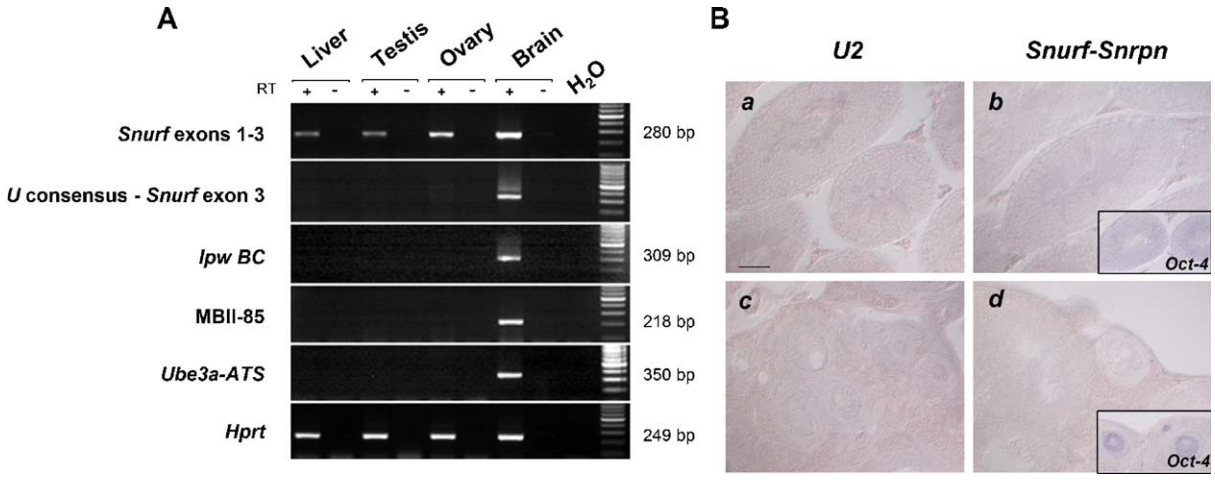


Fig. 6. Expression analyses during mouse gametogenesis. (A) RT-PCR were performed to analyze expression of *LNCAT*, snoRNAs, and *Snurf-Snrpn* in mouse testis and ovary and compare it to their expression in brain and liver. Primer pairs used were as described in Fig. 2A, excepted for the U consensus primer which recognizes the nine exons U described by Landers et al. [Landers, 2004 #78]. RNA integrity was controlled by amplification of an *Hprt* RT-PCR product. (+), with RT; (−), without RT. (B) In situ hybridizations were performed on testis (a, b) and ovary (c, d) with *U2* (a, c) and *Snurf-Snrpn* (b, d) riboprobes. No specific *Snurf-Snrpn* or *U2* expression was detected in oocytes or spermatogenic cells. *Oct-4* expression, used as a positive control in testis and ovaries, was shown in the bottom right hand corner in panels b and d, respectively. Scale bar, 100 μm.

gametogenesis thus leading to active transcription of the paternal expressed genes later in embryo development. We first investigated the expression of *Snurf-Snrpn* and the *LNCAT* in adult ovary and testis by RT-PCR (Fig. 6A). Only *Snurf-Snrpn* is expressed in testis and ovary. We then performed an ISH on testis and ovary sections but we did not detect any *Snurf-Snrpn* hybridization signal in the germ cells (Fig. 6B). Thus, we conclude that the expression of *Snurf-Snrpn* detected by PCR analysis reflects a very low level of expression throughout the testis and the ovary but, in both cases, there is no specific expression in male or female gametes.

Discussion

The investigation of the spatio-temporal regulation/expression of *LNCAT* in vivo is crucial in terms of determining its biological function. We have addressed this issue in regards with the distinct putative roles that had been assigned to *LNCAT* but that had never been investigated.

Our observations allow us to conclude that (1) the *LNCAT* is not a spurious RNA but is developmentally regulated throughout neurogenesis. In addition, we show that *LNCAT* is expressed in postmitotic neurons and that its subcellular localization is mainly cytoplasmic. (2) *LNCAT* is not expressed in the female or male gametes or in early development and, therefore, cannot play a role in the establishment or maintenance of imprinting in the PWS region and (3) the snoRNAs MBII-85 and MBII-52 are constitutively (and not alternatively) spliced from *LNCAT*. Furthermore, we make the novel observation that *LNCAT* and *Snurf-Snrpn* are distinct transcriptional units.

Concerning one *LNCAT* function as *Ube3a-ATS* transcript, we have investigated the expression pattern of *Ube3a-ATS* relative to that of *Ube3a*.We have clearly shown an extensive pattern of expression of *Ube3a-ATS* in neurons where the paternal allele of *Ube3a* is expressed, with a preferential expression of the *Ube3a* maternal allele. We have also observed an expression of *Ube3a-ATS* in structures where *Ube3a* is biallelically expressed (chiasma optic and anterior commissure). More importantly, in those regions where the paternal allele of *Ube3a* is silenced, *Ube3a-ATS* is not detected. Taking into account these and other reported findings, we will discuss different hypotheses to explain the silencing of the paternal allele of *Ube3a* and then propose an alternative model.

LNCAT and Snurf-Snrpn are distinct transcripts but share common exons as well as imprinting and neuronal regulatory elements

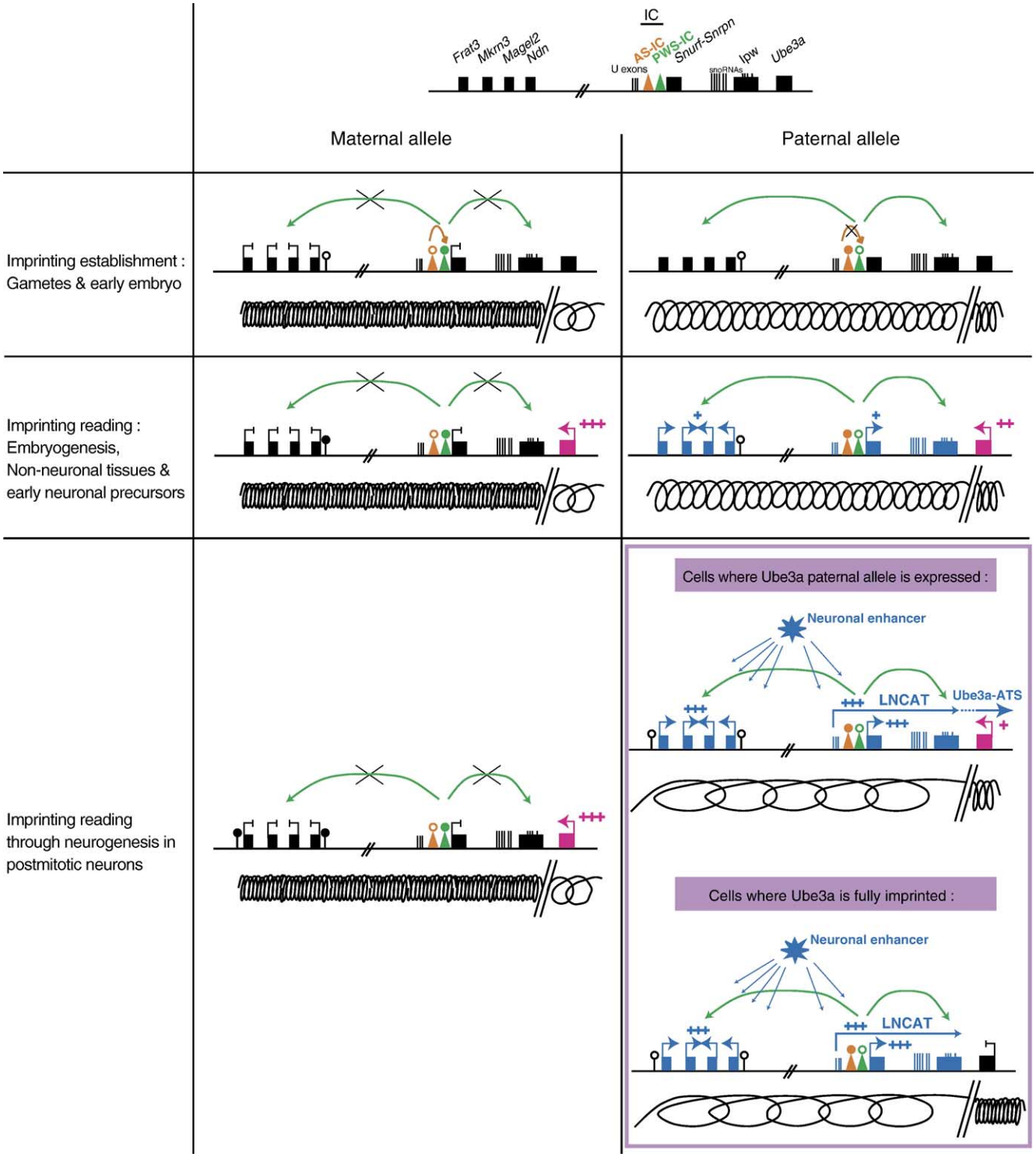
Here, we show that, in vivo, the expression of *LNCAT* is developmentally regulated with expression first detected in the nervous system of 10.5 dpc embryos. *LNCAT* expression is restricted to neurons in the postmitotic territories during embryogenesis. In adult brain, we have also clearly shown a neuronal specific expression of the *LNCAT* exons by colocalization with a neuronal Neurofilament marker.

Snurf-Snrpn transcripts are detected at earlier stages of development, in the embryonic ectoderm. However, from 10.5 dpc, *Snurf-Snrpn* expression is restricted to the nervous system. In adulthood, *Snurf-Snrpn* is expressed ubiquitously with a significant increase in neurons. Overall, the *Snurf-Snrpn* expression pattern during devel-

opment and in adult brain is highly similar to that of *LNCAT* expression pattern. Our data suggest that *LNCAT* and *Snurf-Snrpn* are distinct transcripts and are consistent with our previous suggestion that the U exons initiate the *LNCAT* (Landers et al., 2004). Furthermore, our results strongly suggest that *LNCAT* and *Snurf-Snrpn* transcripts could share regulatory elements such as neuronal enhancers (Fig. 7).

snoRNAs MBII-52 and MBII-85 are constitutively processed from the LNCAT

In adult brain and during embryogenesis, we detect a strong expression of the MBII-52 and MBII-85 snoRNAs in postmitotic neurons, with a pattern that is identical to the *LNCAT* exons (see above). The ISH signal for MBII-52 and MBII-85 is punctate in the nucleus, indicating that we detect



the mature form of these snoRNAs. These data confirm that MBII-52 and MBII-85 are processed from the *LNCAT*, in all the regions where the host transcript is expressed, suggesting a constitutive splicing from this transcript rather than an alternative splicing in a subset of regions where the host RNA is transcribed. The question about the snoRNAs splicing (constitutive or alternative splicing) has not been previously addressed.

The LNCAT and imprinting mechanism

LNCAT is initiated upstream of the imprinting center and encompasses the *Snurf-Snrpn* transcriptional unit, a master regulator of the imprinting process (IC), and overlaps the *Ube3a* transcript. It is conceivable to assign two potential and non-exclusive roles to *LNCAT* in the imprinting mechanism:

1) LNCAT, through the process of transcription itself, could trigger changes in the chromatin structure. Thus, transcription through the IC would activate the chromatin subdomain that includes the paternal "PWS" expressed genes (at least *Snurf-Snrpn*). Such a function for a non-coding RNA has been proposed for the human β-globin locus (Gribnau et al., 2000);
2) LNCAT, including *Ube3a-ATS* exons that overlap *Ube3a*, could play a role in the imprinting (*cis*-silencing) of the paternal *Ube3a* allele.

In terms of the first hypothetical role, we have shown by RT-PCR and ISH that no exon of *LNCAT* is expressed during gametogenesis or in early embryo. These results do not support a role of the *LNCAT* in the imprinting establishment since we would then expect an expression of LNCAT during these stages. We conclude that *LNCAT* is not necessary for imprinting establishment of the "PWS/AS domain" or for *Snurf-Snrpn* expression in early development. It cannot be excluded, however, that *LNCAT* is necessary for modifying or remodeling the chromatin structure in postmitotic neurons thus enabling access of regulatory factors and allowing an upregulation of *Snurf-Snrpn* in the central nervous system.A role for the overlapping *Ube3a* antisense transcript in the silencing of the paternal *Ube3a* allele has been suggested, but not demonstrated, by the observation that the 35-kb deletion of the "PWS-IC" in mouse is associated with loss of *Ube3a-ATS* expression and biallelic expression of *Ube3a* (Chamberlain and Brannan, 2001). However, such a deletion would create a maternal epigenotype in the whole 7C imprinted region preventing the expression of all the "PWS" paternal expressed genes and allowing the paternal allele of *Ube3a* to be expressed as the maternal allele. This model of *Ube3a-ATS* function is based on other examples of antisense-mediated repression (Andersen and Panning, 2003; Wang et al., 2004). Thus, silencing could result from:

1) a post-transcriptional regulation whereby the antisense transcript regulates the sense transcript by an RNAi mechanism.
2) a transcriptional silencing by which the paternal *Ube3a-ATS* transcript itself could recruit an enzymatic complex to silence the chromatin structure. In this situation, RNAi could be involved in recruitment of heterochromatin through the interfering RNAs themselves. It has been shown that dsRNA and proteins of the RNAi machinery can induce transcriptional gene silencing. An alternative way of transcriptional silencing might be that processive transcription of *Ube3a-ATS* could prevent the accessibility to the regulatory elements of *Ube3a* and, possibly, induce a silent chromatin state. We would then expect an exclusive expression of *Ube3a-ATS* in cells where *Ube3A* is silenced and in this case we should never detect both transcripts in the same cell.

Thus, RNAi could regulate the paternal allele of *Ube3a* through a post-transcriptional regulation or even through a transcriptional regulation via heterochromatinization (Matzke and Birchler, 2005). The problem with these two RNAi-mediated silencing mechanisms is that the first step of

Fig. 7. *Cis* regulation of *Ube3a* imprinting model. Genomic imprinting is established during gametogenesis when a primary mark(s) is apposed to the paternal or maternal genome. Kantor et al. proposed that the DNA methylation status of the human AS-IC is the primary imprint (Kantor et al., 2004). On the maternal allele, the AS-IC (orange triangle) is dimethylated (open circles) and thus stimulates methylation (closed circles) of the PWS-IC (green triangle); paternally expressed genes are thus in a closed chromatin conformation. Chromatin structure is represented as a coiled line below the chromosome schematic line and can be found in three different conformations: open (slackened coiled line), semi-open (semi-coiled line), and closed (highly coiled line). On the paternal allele, the AS-IC is methylated and the PWS-IC is correspondingly unmethylated thus allowing an open chromatin state of the PWS genes. As a consequence of the chromatin modification of the PWS domain, the *Ube3a* gene is, at least in early embryo, in an open chromatin structure on the maternal allele but a more closed chromatin could encompass the paternal allele. Later in development, there is a differential reading of the imprint in neuronal and non-neuronal tissues. In non-neuronal cells, PWS genes (*Snurf-Snrpn*, *Necdin*, *Magel2*...) are paternally expressed in a wide range of tissues, but the level of expression is lower than in neuronal tissues. There is a preferential expression of the maternal *Ube3a* allele but the paternal *Ube3a* allele is not silent. During neuronal differentiation, the paternal *Ube3a* allele will be silenced whereas PWS genes, in particular *LNCAT* and *Snurf-Snrpn*, are upregulated most likely by a neuronal enhancer (blue sun shape). The level of expression of the paternal *Ube3a* allele is dependent on the type of *LNCAT* variants in the different neuronal populations. In neurons where *Ube3a-ATS* overlaps *Ube3a*, we can speculate that the process of transcription will enable the paternal *Ube3a* allele to be accessible for transcription factors allowing a low level of transcription. Thus, the *Ube3a-ATS* transcription would enable the paternal expression of *Ube3a*. In those cells where no *LNCAT* variants overlap *Ube3a*, the paternal allele is silenced by specific neuronal factors and/or by the paternal *Ube3a* locus adopting a closed chromatin conformation. In parallel, the maternal allele of *Ube3a* will be accessible to transcription whatever the tissue. The number of plus signs indicates gene expression level. Blue boxes and arrows indicate expression of paternally expressed genes whereas pink boxes and arrows indicate *Ube3a* expression. The slashes (//) on the chromatin scheme indicate the putative chromatin boundaries between the PWS domain and the AS domain (*Ube3a* transcriptional unit).

RNAi is to produce siRNA from a dsRNA corresponding to specific transcripts. This first step of the process requires a Dicer activity. Currently, from what is known in mammals, there is only one DICER and it is present in the cytoplasm (Billy et al., 2001). The recent observation of nuclear RNAi in mammalian cells (Langlois et al., 2005; Robb et al., 2005) is most likely due to the import of diced siRNAs into the nucleus. Since siRNAs are generated in cytoplasm, it is difficult to imagine how only the paternal allele of Ube3a is specifically silenced as we would then expect that both, paternal and maternal, alleles should be targeted.We have performed a detailed analysis of the expression pattern of *LNCAT* variants, especially of *Ube3a-ATS*, in comparison to that of paternal *Ube3a* expression. We have shown that *LNCAT* variants, including *Ube3a-ATS*, are not expressed trough gametogenesis but are expressed throughout mouse development and in adult postmitotic neurons. Particularly, *Ube3a-ATS* is detected in the brain regions, where there is a strong expression bias of the maternal relative to the paternal *Ube3a* allele and in the regions where *Ube3a* displays biallelic expression (Albrecht et al., 1997; Jiang et al., 1998). Absence of *Ube3a-ATS*, however, occurs only in cells where paternal *Ube3a* is silenced.The absence of full-length transcript from germ cells and neurons does not mean there is no siRNA (in fact, absence of long transcripts would be consistent with siRNA presence), so that a role for siRNA derived from the ncRNA cannot be excluded. However, our data are not readily compatible with one of the aforementioned hypotheses to explain how *Ube3a-ATS* could silence the paternal *Ube3a* allele.In order to integrate our novel findings and data from the literature, we propose a new alternative model for the role of *LNCAT* in imprinting regulation (Fig. 7).

A new model for Ube3a-ATS function

There are several observations that argue against the role of LNCAT in imprinting regulation. The first is our demonstration that the transcription of *Ube3a-ATS* is detected in cells where the paternal allele of *Ube3a* is expressed but not in neurons where paternal allele of *Ube3a* is silenced. Next, Rougeulle et al. have shown an enrichment of H3K4 dimethylation within the *Ube3a* promoter region (Rougeulle et al., 2003). This type of chromatin modification appears to be characteristic of other monoallelic expressed genes including *IGF2R*, a gene that displays tissue-specific imprinting (Vu et al., 2004). The enrichment of H3K4 dimethylation in the Ube3a promoter is observed in ES cells even though *Ube3a* is biallelically expressed in this cell type. A study performed by RNA-FISH indicated preferential maternal *UBE3A* expression in human non-neuronal tissues including fibroblasts, lymphoblasts, and undifferentiated neuronal cells (Herzing et al., 2002), where *UBE3A-ATS* is not expressed (Runte et al., 2004). Altogether, these observations suggest that, in early development, *Ube3a* alleles are in a differential chromatin structure and are differentially expressed depending on their parental origin. In this model (Fig. 7), we propose that imprinting of the 7c locus is established by a differential chromatin structure during gametogenesis and/or early development. The paternal *Ube3a* allele is thereby predisposed to be silenced whereas the maternal allele is in an open chromatin structure. During neurogenesis, the paternal genes are upregulated and the chromatin structure of PWS domain is in an active state. As neurogenesis proceeds, paternal *Ube3a* expression is dependent on the type of *LNCAT* variants in the different neuronal populations. In neurons where *Ube3a-ATS* overlaps *Ube3a*, the process of antisense transcription permits the accessibility of regulatory factors and enables a low level of paternal *Ube3a* transcription as it is observed. In those cells where no *LNCAT* variants overlap *Ube3a*, the paternal allele is silenced by specific neuronal factors and/or by the paternal *Ube3a* locus adopting a closed chromatin conformation. The novel implication of this model is that *Ube3a-ATS* transcription enables the paternal expression of *Ube3a*.

The precise role of LNCAT in Ube3a regulation awaits further confirmation. In this regard, the introduction of polyA signal to block LNCAT transcription has been attempted (M.L., personal communication), but proved unsuccessful, probably because of the complex pattern of alternative promoters and alternative splicing of *LNCAT*. While a functional approach is needed, our study represents one of the few approaches that can validate or not the proposed roles for *LNCAT* and potentially reveal novel functions of this non-coding antisense transcript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.07.030.

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